(19) Europäisches Patentamt
European Patent Offic
Office européen des brev ts

(11) EP 1 055 684 A1

(12) **EUROPEAN PATENT APPLICATION**
published in accordance with Art. 158(3) EPC

(43) Date of publication:
29.11.2000 Bulletin 2000/48

(21) Application number: **98957127.8**

(22) Date of filing: **02.12.1998**

(51) Int. Cl.[7]: **C07K 7/06**, C12N 5/08, C12N 15/12, A61K 38/08, A61K 45/05

(86) International application number:
PCT/JP98/05430

(87) International publication number:
WO 99/29715 (17.06.1999 Gazette 1999/24)

(84) Designated Contracting States:
AT BE CH DE DK ES FI FR GB GR IE IT LI NL PT SE

(30) Priority: **05.12.1997 JP 33574597**

(71) Applicant: **Itoh, Kyogo**
Miyaki-gun, Saga 841-02 (JP)

(72) Inventors:
- **ITOH, Kyogo**
Miyaki-gun, Saga 841-0205 (JP)
- **SHICHIJO, Shigeki**
Kurume-shi, Fukuoka 830-0003 (JP)
- **IMAI, Yasuhisa**
Kurume-shi, Fukuoka-ken 830-0012 (JP)

(74) Representative:
VOSSIUS & PARTNER
Siebertstrasse 4
81675 München (DE)

(54) TUMOR ANTIGEN PEPTIDE DERIVATIVES

(57) Tumor antigen peptide derivatives which comprise all or part of an amino acid sequence derived from the amino acid sequence shown in SEQ ID NO: 3 through the alteration of one to several amino acid residues, which are capable of binding to HLA-A24 antigen and thus being recognized by cytotoxic T cells; the use of these tumor antigen peptide derivatives in treating, preventing and diagnosing tumors; and remedies or preventives for tumors containing these peptide derivatives as an active ingredient.

EP 1 055 684 A1

Printed by Xerox (UK) Business Services
2.16.7 (HRS)/3.6

Description

TECHNICAL FIELD

[0001] The present invention relates to novel tumor antigen peptide derivatives.

BACKGROUND ART

[0002] It is known that the immune system, particularly T cells, plays an important role *in vivo* in tumor rejection. Indeed, infiltration of lymphocytes having cytotoxic effects on tumor cells has been observed in human tumor foci (*Arch. Surg.*, **126**:200-205, 1990), and cytotoxic T lymphocytes (CTLs) recognizing autologous tumor cells have been isolated from melanomas without great difficulties (e.g., *Immunol. Today*, **8**:385, 1987; *J. Immunol.*, **138**:989, 1987; and *Int. J. Cancer*, **52**:52-59, 1992). In addition, the results of clinical treatment of melanomas by T cell introduction also suggest the importance of T cells in tumor rejection (*J. Natl. Cancer. Inst.*, **86**:1159, 1994).

[0003] Although it has long been unknown about target molecules for CTLs attacking autologous tumor cells, the recent advance in immunology and molecular biology has gradually revealed such target molecules. Specifically, it has been found that using T cell receptors (TCRs), CTL recognizes a complex between a peptide, called tumor antigen peptide, and a major histocompatibility complex class I antigen (MHC class I antigen, and in the case of human, referred to as HLA antigen), and thereby attacks autologous tumor cells.

[0004] Tumor antigen peptides are generated from proteins specific for tumors, that is, tumor antigen proteins. Thus, the proteins are intracellularly synthesized and then degraded in cytoplasm into the peptides by proteasome. On the other hand, MHC class I antigens (HLA antigens) formed at endoplasmic reticulum, when bind to the above tumor antigen peptides, are transported via cis Golgi to trans Golgi, *i.e.*, the mature side and carried to the cell surface where they are presented as an antigen. A tumor-specific CTL recognizes this complex presented as an antigen, and exhibits its anti-tumor effects through the cytotoxic effect or production of lymphokines (*Rinsho-Menneki*, **27**(9):1034-1042, 1995). As a consequence of such elucidation of a series of actions, it has become possible to treat tumors by using tumor antigen proteins or tumor antigen peptides as so-called cancer vaccines which enhance tumor-specific CTLs in a patient.

[0005] As such tumor antigen proteins, T. Boon *et al.* identified a protein named MAGE from human melanoma cells for the first time in 1991 (*Science*, **254**:1643-1647, 1991), and thereafter several additional tumor antigen proteins have been identified from melanoma cells.

[0006] As reviewed by T. Boon *et al.* (*J. Exp. Med.*, **183**, 725-729, 1996), tumor antigen proteins hitherto identified can be divided into the following four categories.

[0007] Tumor antigen proteins belonging to the first category are those which are expressed in testis only as normal tissues, while they are expressed in melanoma, head and neck cancer, non-small cell lung cancer, bladder cancer and others, as tumor tissues. Among tumor antigen proteins in this category are the above-described MAGE and analogous proteins constituting a family of more than 12 members (*J. Exp. Med.*, **178**:489-495, 1993), as well as BAGE (*Immunity*, **2**:167-175, 1995) and GAGE (*J. Exp. Med.*, **182**:689-698, 1995), all of which have been identified in melanoma cells.

[0008] Although some of such tumor antigen proteins in this category are highly expressed in melanoma, the expression thereof is observed in only 10 to 30% of patients having a particular tumor other than melanoma, and therefore, they cannot be applied widely to treatments or diagnoses of various tumors.

[0009] Tumor antigen proteins belonging to the second category are those which are expressed only in melanocytes and retina among normal tissues, and in melanomas among tumor tissues. Since these tissue-specific proteins are highly expressed in melanomas, they would function as tumor antigen proteins specific for melanomas. Among tumor antigen proteins in this category are tyrosinase (*J. Exp. Med.*, **178**:489-495, 1993), MART-1 (*Proc. Natl. Acad. Sci. USA*, **91**:3515, 1994), gp100 (*J. Exp. Med.*, **179**:1005-1009, 1994), and gp75 (*J. Exp. Med.*, **181**:799-804, 1995). Genes encoding these proteins have all been cloned from melanoma cells. Melan-A (*J. Exp. Med.*, **180**:35, 1994), which has been separately isolated, has proved to be identical with MART-1.

[0010] However, the tumor antigen proteins of this category cannot be used widely in the treatments or diagnoses of various tumors, since they are not expressed in tumors other than melanoma.

[0011] Tumor antigen proteins belonging to the third category are those which are expressed as tumor antigen peptides recognized by CTL as a result of tumor-specific mutations. Among tumor antigen proteins in this category are mutated CDK4 (*Science*, **269**:1281-1284, 1995), β-catenin (*J. Exp. Med.*, **183**:1185-1192, 1996), and MUM-1 (*Proc. Natl. Acad. Sci. USA*, **92**:7976-7980, 1995). In CDK4 and β-catenin, a single amino acid mutation increases the binding affinity of the peptides to MHC class I antigen, which allows them to be recognized by T cells. In MUM-1, an intron, which normally is not translated, is translated due to mutation, and the resultant peptide is recognized by T cells. However, since such mutations occur at low frequency, they cannot be applied widely to treatments or diagnoses of various tumors.

[0012] Tumor antigen proteins belonging to the fourth category are those widely expressed in normal tissues and also recognized by CTL, example of which includes P15 identified from melanoma cells (*J. Immunol.* **154**:5944-5955, 1995)

[0013] Tumor antigen proteins or peptides hitherto known have been identified in the following manners.

[0014] A set of tumor cell and CTL attacking the tumor cell (usually established from lymphocytes of the same patient from whom the tumor cells are obtained) is first provided. Then, the set of cells are used to directly identify tumor antigen peptides, or to determine a gene encoding tumor antigen protein, from which the corresponding tumor antigen peptide is identified.

[0015] In the method where tumor antigen peptides are directly identified, tumor antigen peptides bound to MHC class I antigens in tumor cells are extracted under acidic conditions, and separated into various peptides using high-performance liquid chromatography. Tumor antigen peptides are then identified by pulsing cells expressing MHC class I antigen but not expressing tumor antigen protein (for example, B cells from the same patient) with the resultant peptides and examining the reactivity with CTL. The sequences of the peptides thus identified are then determined by, for example, mass spectrometry. In this way, tumor antigen peptides derived from Pmel 17 which is the same molecule as gp100 have been identified from melanoma cells (*Science*, **264**:716-719, 1994).

[0016] In the method where a gene encoding tumor antigen protein is first obtained which is followed by the identification of the corresponding tumor antigen peptide, such a gene can be cloned using molecular biological techniques. MHC class I antigen gene and cDNAs prepared from tumor cells are co-transfected into cells not expressing tumor antigen proteins (for example, COS cells) for transient expression. The expression products are then repeatedly screened on the basis of their reactivity with CTL to isolate a gene encoding tumor antigen protein. In this way, the genes encoding the above-mentioned MAGE, tyrosinase, MART-1, gp100, and gp75 have been cloned.

[0017] The following method can be used to actually deduce and identify a tumor antigen peptide bound to and presented with MHC class I antigen (HLA antigens) on the basis of information about tumor antigen gene. Fragments of various sizes are first prepared from a gene encoding tumor antigen protein by means of PCR, exonucleases, or restriction enzymes, or the like, and cotransfected with MHC class I antigen gene into cells not expressing tumor antigen proteins (e.g., COS cells) for transient expression. The region(s) which include tumor antigen peptides are then identified on the basis of their reactivity with CTL. Subsequently, peptides are synthesized on the basis of the identified regions. Cells expressing MHC class I antigen but not expressing tumor antigen proteins are then pulsed with the synthesized peptides to identify the tumor antigen peptides, for example, by examining their reactions with CTL (*J. Exp. Med.*, **176**:1453, 1992; *J. Exp. Med.*, **179**:24, 759, 1994). The sequence regularities (motifs) for peptides, which are bound and presented by certain types of MHC such as HLA-A1, -A0201, -A0205, -A11, -A24, -A31, -A6801, -B7, -B8, - B2705, -B37, -Cw0401, and -Cw0602 have been known (*Immunogenetics*, **41**:178-228, 1995), and therefore, candidates for tumor antigen peptides may also be designed by making reference to such motifs, synthesized and examined in the same way as described above (*Eur. J. Immunol.*, **24**:759, 1994; *J. Exp. Med.*, **180**:347, 1994).

[0018] According to procedures as described above, various tumor antigen proteins and tumor antigen peptides have been hitherto identified. As described above, however, some of the known tumor antigen proteins are expressed only in limited tumors, and others are expressed only in a small number of patients having a particular tumor even if they are expressed in various kinds of tumor, and therefore, they cannot be used widely in the treatments or diagnoses of various tumors.

DISCLOSURE OF INVENTION

[0019] One of the purposes of the present invention is to provide tumor antigen peptide derivatives which can be used widely and universally without limitations regarding the kind of tumor or the subjects, in particular, tumor antigen proteins, tumor antigen peptides corresponding thereto, and derivatives thereof which can be widely applied to treatments and diagnoses of tumors with high incidence such as squamous cell carcinoma. Thus, it is one of purposes of the present invention to provide novel tumor antigen peptide derivatives derived from a tumor other than melanomas, in particular, from a squamous cell carcinoma, and also methods, compositions, kits, and the like, for treating, preventing or diagnosing tumors by the use of said tumor antigen peptide derivatives. It is also a purpose of the present invention to provide tumor antigen peptide derivatives restricted to HLA-A24, which is an HLA antigen carried by a large part of human subjects.

[0020] To this end, the present inventors have established a squamous cell carcinoma cell line KE-4 derived from esophageal cancer (hereinafter referred to as esophageal cancer cell line KE-4 or simply as KE-4), and also established CTL (hereinafter referred to as KE-4CTL) which recognizes tumor antigen peptides restricted to HLA antigens such as HLA-A2601, HLA-A2402, and the like, which are expressed by said KE-4 (*Cancer Res.*, **55**:4248-4253, 1995).

[0021] Fibroblast cell line VA-13 was then cotransfected with a recombinant plasmid of cDNA library prepared from KE-4 and a recombinant plasmid containing HLA-A2601 cDNA. The screening of a gene(s) encoding novel tumor antigen protein was carried out by treating the resulting transfectants with KE-4CTL, and measuring the amount of pro-

duced IFN-γ to determine whether KE-4-CTL was activated or not. As a result, the inventors succeeded in cloning a novel gene encoding a novel tumor antigen protein for the first time from tumor cells other than melanomas. The nucleotide sequence of the cloned gene is shown in SEQ ID NO: 2, and the deduced amino acid sequence is shown in SEQ ID NO: 1.

[0022] Subsequently, the present inventors tried to identify the portions in the amino acid sequence of the above tumor antigen protein that actually function as tumor antigen peptides, and identified various tumor antigen peptide portions restricted to HLA-A26, HLA-A24, and the like.

[0023] Among them, a peptide having the amino acid sequence at positions 690 to 698 (SEQ ID NO: 3) in the amino acid sequence shown in SEQ ID NO: 1 was identified as an HLA-A24-restricted tumor antigen peptide. The present inventors then prepared various peptide derivatives by altering an amino acid residue(s) in the HLA-A24-restricted tumor antigen peptide shown in SEQ ID NO: 3 and determined their activities, which revealed that the derivatives also have the activity as a tumor antigen peptide.

[0024] The present invention was completed on the basis of such findings.

[0025] Thus, the gist of the present invention is to provide tumor antigen peptide derivatives which comprise all or part of an amino acid sequence wherein one to several amino acid residues in the amino acid sequence shown in SEQ ID NO: 3 are altered, and which derivatives are capable of binding to HLA-A24 antigen and thus being recognized by cytotoxic T cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026]

Fig. 1 depicts electrophoretograms showing the result of analysis of distribution of tumor antigen protein mRNA expression in various cell lines (a) and various tissues (b), including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, uterus, small intestine, and colon (mucosal lining), as well as in peripheral blood leukocyte, by Northern blot hybridization using, as a DNA probe, the inserted sequence portion in a recombinant plasmid 6DI that encodes the tumor antigen protein cloned from the esophageal cancer cell line KE-4.

In Fig. 1 a), KE-4, KE-3, TE-8, and TE-9 indicate esophageal cancer cell lines; Kuma-1 indicates a head and neck cancer cell line; HSC-4 indicates a mouth cancer cell line; Bec-1 indicates a B cell line; KMG-A indicates a gallbladder cancer cell line; R-27 indicates a breast cancer cell line; KIM-I, KYN-1, and HAK-3 indicate hepatic cancer cell lines; and M36 and M37 indicate melanoma cell lines. From Fig. 1, it can be seen that the mRNA for the tumor antigen protein encoded in the clone 6DI is widely expressed in various cancer cells and normal tissues.

Fig. 2 depicts a bar graph showing the *in vitro* IFN-γ-inducing activities of peptides having the amino acid sequences shown in SEQ ID NOs: 5, 6, and 7. Specifically, peripheral blood lymphocytes from an HLA-A24-positive healthy individual were stimulated with the above peptide derivatives, and the amounts of IFN-γ produced by the stimulated lymphocytes were measured in the presence of HLA-A24-positive KE-4 cells expressing the tumor antigen. It can be seen from Fig. 2 that CTLs are induced by each peptide derivative of SEQ ID NOs: 5, 6, and 7.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0027] In this specification, "tumor antigen peptide derivatives" of the present invention refer to those which comprise all or part of an amino acid sequence wherein one or more amino acid residues and preferably one to several amino acid residues in the amino acid sequence shown in SEQ ID NO: 3 are altered, and which have the activity as a tumor antigen peptide, i.e., the ability to bind to HLA-A24 antigen and be recognized by CTL. A peptide having the amino acid sequence of SEQ ID NO: 3 is an HLA-A24 restricted tumor antigen peptide which is located at amino acid No. 690 to 698 of the amino acid sequence of the tumor antigen protein shown in SEQ ID NO: 1.

[0028] Accordingly, all the peptides that contain all or part of a derivative wherein one or more amino acid residues in the amino acid sequence of the tumor antigen peptide shown in SEQ ID NO: 3 are altered and have the activity as a tumor antigen peptide, i.e., which peptides are capable of binding to HLA-A24 antigen and thus being recognized by CTL, fall within the scope of tumor antigen peptide derivatives of the present invention.

[0029] In the present invention, "capable of binding to HLA-A24 antigen and thus being recognized by CTL" means that the tumor antigen peptide derivative can bind to HLA-A24 antigen to form a complex and that CTL can recognize such complex.

[0030] In the present invention, the "alteration" of amino acid residues means substitution, deletion and/or addition of amino acid residues, and a preferred example is substitution of amino acid residues. The following descriptions mainly concern substitution of amino acid residues, but the same descriptions are also applicable to deletion or addition of amino acid residues.

[0031] Tumor antigen peptide derivatives of the present invention can be identified, for example, by synthesizing a candidate peptide which comprises all or part of an amino acid sequence wherein one or more, preferably one to several, amino acid residues in the amino acid sequence shown in SEQ ID NO: 3 are substituted with other amino acid residues, and then conducting an assay for determining whether or not the complex between said candidate peptide and HLA-A24 antigen is recognized by CTL.

[0032] Although the number and the position of amino acid residues to be substituted may be determined arbitrarily so long as the activity as a tumor antigen peptide is retained, it is preferred that one to several residues are substituted since the peptide fragment shown in SEQ ID NO: 3 consists of nine amino acid residues.

Synthesis of peptides

[0033] Synthesis of peptides may be achieved by the methods usually used in peptide chemistry, for example, those described in the literatures such as "Peptide Synthesis", Interscience, New York, 1966; "The Proteins", vol. 2, Academic Press Inc., New York, 1976; "Pepuchido-Gosei", Maruzen, 1975; "Pepuchido-Gosei-no-Kiso-to-Jikkenn", Maruzen, 1985; and "Iyakuhin-no-Kaihatu, Zoku, vol. 14, Peputido-Gosei", Hirokawa Shoten, 1991.

Recognition by HLA-antigen-restricted CTL

[0034] It can be examined whether or not a synthesized candidate peptide is capable of binding to HLA-A24 antigen and thus being recognized by CTL, for example, by the following methods.

(1) According to the method described in *J. Immunol.*, **154**:2257 (1995), peripheral blood lymphocytes are isolated from an HLA-A24 antigen-positive human, and it is determined whether CTL that specifically recognizes HLA-A24-positive cells pulsed with the candidate peptide is induced or not, when the lymphocytes are stimulated *in vitro* by adding the candidate peptide. The presence or absence of CTL induction may be determined, for example, by measuring the amounts of various cytokines (for example, IFN-γ) produced by CTL in response to the antigen peptide-presenting cells using an enzyme-linked immunosorbent assay (ELISA) or the like. Alternatively, a method in which the cytotoxicity of CTL against antigen peptide-presenting cells labeled with ^{51}Cr is measured (^{51}Cr release assay, *Int. J. Cancer*, **58**:317, 1994) may also be used. HLA-A24-positive cells used in the above assays may be generally available cells such as an esophageal cancer cell line KE-4 (FERM BP-5955) or SKG-IIIa cells (JCRB 0232).

(2) Furthermore, the examination can also be done by introducing an HLA-A24 cDNA expressing plasmid into COS-7 cells (ATCC No. CRL1651) or VA-13 cells (RIKEN CELL BANK, The Institute of Physical and Chemical Research), pulsing the obtained cells with the above candidate peptide, reacting them with KE-4CTL (Deposit Number: FERM BP-5954), an HLA-A24-restricted CTL line, and then measuring the amounts of various cytokines (for example, IFN-γ) produced by KE-4CTL (*J. Exp. Med.*, **187**:277, 1998).

[0035] Illustrative examples of various assay as described above are shown in Reference Examples 7 and 8 as well as Example 2 below.

[0036] In addition, the binding affinity of a tumor antigen peptide derivative to HLA-A24 antigen may easily be measured in a cell-free system using a competitive inhibition assay for binding to the HLA antigen between said derivative and the standard peptide (SEQ ID NO: 3) labeled with a radioisotope (R. T. Kubo *et al.*, *J. Immunol.*, **152**:3913, 1994).

[0037] Length of a tumor antigen peptide derivative of the present invention is not specifically restricted provided that it binds to HLA-A24 antigen and is thus recognized by CTL. According to the purposes of the present invention, tumor antigen peptide derivatives of the present invention include those not only presented by themselves on the surface of antigen-presenting cells after binding to HLA-A24 antigen, but also those fragmented as appropriate within target cells to yield peptide fragments of an appropriate length, which fragments comprise all or part of an amino acid sequence wherein one to several amino acid residues in the amino acid sequence of SEQ ID NO: 3 are altered and are capable of binding to HLA-A24 antigen and thus being recognized by CTL.

[0038] Preferably, a peptide fragment that in itself binds to HLA-A24 antigen and is thus presented has a length of 8 to 11 amino acids. Accordingly, examples of peptide derivatives obtainable by amino acid substitution include 1) a peptide consisting of 9 amino acids having an amino acid sequence in which one to several amino acid residues in the amino acid sequence shown in SEQ ID NO: 3 are substituted by other amino acid residue(s); or 2) a peptide of about 10 to 11 amino acids in length that comprises the whole peptide of the above 1) or a peptide consisting of about 8 amino acids that comprises part of the peptide of the above 1), wherein the derivatives retain the tumor antigen peptide activity of binding to HLA-A24 antigen and thus being recognized by CTL.

[0039] Intended tumor antigen peptide derivatives can be obtained by synthesizing various peptides in which an

amino acid or amino acids at any position(s) in the amino acid sequence of SEQ ID NO: 3 are altered, and screening on the basis of the activity as a tumor antigen peptide according to the descriptions in the present specification.

[0040] There are certain rules (motifs) in the sequences of antigen peptides bound and presented by HLA antigen. Concerning the motif for HLA-A24, it is known that in the sequence of peptides consisting of 8 to 11 amino acids, the amino acid at the second position from the N-terminus is phenylalanine, tyrosine, methionine, or tryptophan, and the C-terminal amino acid is phenylalanine, leucine, isoleucine, tryptophan, or methionine (*Immunogenetics,* **41**:178-228, 1995; *J. Immunol.*, **152**:3913, 1994; *J. Immunol.*, **155**:4307, 1994). In addition, derivatives obtainable by substituting an amino acid consistent with such motif with another amino acid having analogous properties may also potentially be accepted as HLA-A24 antigen binding peptide.

[0041] Accordingly, examples of tumor antigen peptide derivative of the present invention include a peptide derivative that comprises all or part of an amino acid sequence wherein the amino acid residue(s) at the second position and/or the ninth position (the C-terminus) in the amino acid sequence shown in SEQ ID NO: 3 are substituted by other amino acid residues and that has the activity of binding to HLA-A24 antigen and thus being recognized by CTL.

[0042] Thus, the present invention provides a tumor antigen peptide derivative that comprises all or part of an amino acid sequence wherein the amino acid residue(s) at the second position and/or the ninth position in the amino acid sequence shown in SEQ ID NO: 3 are substituted by other amino acid residues and that is capable of binding to HLA-A24 antigen and thus being recognized by CTL.

[0043] In preferred peptide derivatives, the amino acid residue(s) at the second position and/or the ninth position in the amino acid sequence shown in SEQ ID NO: 3 are substituted by an amino acid residue consistent with the above motif Specifically, preferred tumor antigen peptide derivatives are those comprising all or part of an amino acid sequence wherein tyrosine at the second position in the amino acid sequence shown in SEQ ID NO: 3 is substituted by phenylalanine, methionine, or tryptophan and/or phenylalanine at the ninth position is substituted by leucine, isoleucine, tryptophan, or methionine, and having the activity described above, which amino acid sequence is shown in SEQ ID NO: 4.

[0044] Thus, in another embodiment, the present invention provides a tumor antigen peptide derivative comprising all or part of the amino acid sequence shown in SEQ ID NO: 4 and being capable of binding to HLA-A24 antigen and thus being recognized by CTL.

[0045] Furthermore, preferred examples of a tumor antigen peptide derivative that contains substitution of amino acid residue according to the above motif are tumor antigen peptide derivatives that comprise all or part of an amino acid sequence wherein phenylalanine at the ninth position in the amino acid sequence shown in SEQ ID NO: 3 is substituted by tryptophan, leucine, or isoleucine; tumor antigen peptide derivatives that comprise all or part of an amino acid sequence wherein tyrosine at the second position in the amino acid sequence shown in SEQ ID NO: 3 is substituted by phenylalanine; and tumor antigen peptide derivatives that contain a combination of such substitutions.

[0046] Accordingly, in a preferred embodiment, the present invention provides a tumor antigen peptide derivative comprising all or part of an amino acid sequence wherein phenylalanine at the ninth position in the amino acid sequence shown in SEQ ID NO: 3 is substituted by tryptophan, leucine, or isoleucine and being capable of binding to HLA-A24 antigen and thus being recognized by CTL.

[0047] In a further preferred embodiment, the present invention provides a tumor antigen peptide derivative comprising all or part of an amino acid sequence wherein tyrosine at the second position in the amino acid sequence shown in SEQ ID NO: 3 is substituted by phenylalanine and being is capable of binding to HLA-A24 antigen and thus being recognized by CTL.

[0048] In a still further preferred embodiment, the present invention provides a tumor antigen peptide derivative comprising all or part of an amino acid sequence wherein phenylalanine at the ninth position in the amino acid sequence shown in SEQ ID NO: 3 is substituted by tryptophan, leucine, or isoleucine, and tyrosine at the second position is also substituted by phenylalanine, and being capable of binding to HLA-A24 antigen and thus being recognized by CTL.

[0049] A particularly preferred tumor antigen peptide derivative comprises all or part of the amino acid sequence shown in SEQ ID NO: 5.

[0050] Tumor antigen peptide derivatives of the present invention bind to HLA-A24, which is an HLA antigen found in a large part of human subjects (for example, in about 60% of Japanese), and presented thereby. Accordingly, the present derivatives are expected to be useful as novel anti-tumor agents, since they are generally usable in most of tumor patients and also widely applicable to tumors of high incidence such as squamous cell carcinoma. In this connection, squamous cell carcinoma is one of human cancers that are most frequently found, and particularly esophageal cancer and lung cancer are known to be relatively resistant to current chemotherapy or radiotherapy.

[0051] As described below in detail, tumor antigen peptide derivatives of the present invention are useful *in vivo* and *in vitro* for various purposes including treatment, prophylactic, or diagnosis of tumors.

[0052] Thus, the present invention also provides a therapeutic or prophylactic agent for tumors that comprises as an active ingredient at least one of the above-described tumor antigen peptide derivatives of the present invention.

[0053] When used with the aim of treating or preventing tumors, at least one of, or a combination of two or more of, tumor antigen peptide derivatives of the present invention is administered to a patient, if necessary, in combination with, for example, other tumor antigen peptides. When a therapeutic or prophylactic agent for tumors of the present invention is administered to a patient who is HLA-A24-positive and is also positive in respect to the tumor antigen protein from which the tumor antigen peptide derivatives of the present invention are derived, the said peptide derivative is presented at high density with HLA-A24 antigen of antigen-presenting cells, then CTL specific for the presented HLA-A24 antigen complex proliferates and destroys tumor cells. As a result, the tumor of the patient may be treated, or proliferation or metastasis of the tumor may be prevented. As described above, the tumor antigen protein from which tumor antigen peptide derivatives of the present invention are derived is widely expressed, for example, on squamous cell carcinomas such as esophageal cancer, lung cancer, and the like. A therapeutic or prophylactic agent for tumors of the present invention has therefore an advantage of having a wide range of application. Furthermore, although the above squamous cell carcinomas often exhibit resistance to chemotherapy and radiotherapy, a combined use of a therapeutic agent for tumors of the present invention make it possible to achieve a desired therapeutic effect. In addition, it is also a great advantage that treatment can be given without specifying the site at which the cancer develops.

[0054] A therapeutic or prophylactic agent for tumors containing a tumor antigen peptide derivative of the present invention may be administered along with an adjuvant in order to effectively establish the cellular immunity, or may be administered in a particulate dosage form. For such purpose, those adjuvants described in the literature (*Clin. Microbiol. Rev.*, **7**:277-289, 1994) are applicable. In addition, dosage forms which allow the foreign antigen peptide derivatives to be efficiently presented on HLA antigen, such as liposomal preparations, particulate preparations in which the derivatives are bound to beads having a diameter of several μm, or preparations in which the derivatives are attached to lipids, are also used. Administration may be achieved, for example, intradermally, hypodermically, by intravenous injection, or the like. Although the dose of a tumor antigen peptide derivative of the present invention administered may be adjusted as appropriate depending on, for example, the disease to be treated, the age and the body weight of a particular patient, it would be usually from 0.0001 mg to 1000 mg, preferably 0.001 mg to 1000 mg, and more preferably 0.1 mg to 10 mg of the derivative every several days to every several months.

[0055] Furthermore, tumor antigen peptide derivatives of the present invention can be used for *in vitro* induction of antigen-presenting cells and such cells are useful in, for instance, the treatment of tumors.

[0056] Accordingly, the present invention provides an antigen-presenting cell which comprises a complex between HLA-A24 antigen and a tumor antigen peptide derivative of the present invention, the complex being presented on the surface of an isolated cell which has an antigen-presenting ability and is derived from a tumor patient.

[0057] The present invention further provides a therapeutic agent for tumors which comprises the above antigen-presenting cells as an active ingredient.

[0058] Although the "cell having an antigen-presenting ability" is not specifically restricted to any cell so long as it is expressing HLA-A24 antigen capable of presenting a tumor antigen peptide derivative of the present invention on the surface, dendritic cells, which is reported to have an especially high antigen-presenting ability, are preferred.

[0059] In order to prepare such antigen-presenting cells, cells having an antigen-presenting ability are isolated from a tumor patient, and pulsed *ex vivo* with a tumor antigen peptide derivative of the present invention to form a complex between HLA-A24 antigen and the peptide derivative (*Cancer Immunol. Immunother.*, **46**:82, 1998).

[0060] A therapeutic agent for tumors that comprises the above antigen-presenting cells as an active ingredient preferably contains physiological saline, phosphate buffered saline (PBS), culture medium, or the like in order to stably maintain the antigen-presenting cells. Administration may be achieved, for example, intravenously, hypodermically, or intradermally. By returning the above therapeutic agent for tumors into the patient's body, specific CTL is efficiently induced in the patient who is HLA-A24 positive and is also positive for the tumor antigen protein from which tumor antigen peptide derivatives of the present invention are derived. The tumor can be thereby treated, and furthermore its metastasis may also be prevented.

[0061] In addition, another example of *in vitro* use of tumor antigen peptide derivatives of the present invention may be in the following adoptive immunotherapy.

[0062] In the case of melanoma, it has been observed that an adoptive immunotherapy wherein intratumoral T cell infiltrate taken from the patient himself/herself are cultured *ex vivo* in large quantities, and then returned into the patient achieves an therapeutic gain (*J. Natl. Cancer. Inst.*, **86**:1159, 1994). Furthermore, in mouse melanoma, suppression of metastasis has been observed by stimulating splenocytes *in vitro* with a tumor antigen peptide TRP-2, thereby proliferating CTLs specific for the tumor antigen peptide, and then administering said CTLs into a mouse carrying grafted melanoma (*J. Exp. Med.*, **185**:453, 1997). This resulted from *in vitro* proliferation of CTL that specifically recognizes the complex between an HLA antigen of antigen-presenting cells and the tumor antigen peptide. A method for treating tumors which comprises *in vitro* stimulating peripheral blood lymphocytes from a patient with a tumor antigen peptide derivative of the present invention to proliferate tumor-specific CTLs, and returning the CTLs into the patient is believed to be useful.

[0063] Accordingly, the present invention also provides cytotoxic T cells that specifically recognize a complex

between HLA-A24 antigen and the above tumor antigen peptide derivative.

[0064] Furthermore, the present invention provides a therapeutic agent for tumor which comprises the above cytotoxic T cells as an active ingredient.

[0065] It is preferred that the therapeutic agent contains physiological saline, phosphate buffered saline (PBS), culture medium, or the like in order to stably maintain CTLs. Administration may be achieved, for example, intravenously, hypodermically, or intradermally. By returning the above therapeutic agent into the patient's body, the toxic effect of CTLs on tumor cells is enhanced in the patient who is HLA-A24 positive and is also positive for the tumor antigen protein from which tumor antigen peptide derivatives of the present invention are derived. The destruction of tumor cells leads to the treatment of tumor and the prevention of metastasis.

[0066] To use a tumor antigen peptide derivative of the present invention in diagnosis of tumors, for example, an antibody against the tumor antigen peptide derivative is prepared in a conventional manner and labeled appropriately, if necessary. The antibody is used to detect the presence of the antigen in a sample (such as blood, a tumor tissue, or the like) obtained from a patient suspected to have a tumor, thereby diagnosing the presence or absence of tumors. A tumor antigen peptide derivative of the present invention itself can also be used as a diagnostic agent for detecting the presence of antibody in the above-mentioned sample such as blood or tumor tissue to diagnose the presence or absence of tumors.

[0067] The present invention also provides a method of treating, preventing, or diagnosing tumors using a tumor antigen peptide derivative described above, antigen-presenting cells that are presenting said tumor antigen peptide derivative, cytotoxic T cells that specifically recognize a complex between said tumor antigen peptide derivative and HLA-A24 antigen. Furthermore, tumor antigen peptide derivatives of the present invention are also useful as research reagents.

[0068] The following Examples are provided to further illustrate the present invention and are not to be construed as limiting the scope thereof.

Reference Example: Cloning of a tumor antigen protein cDNA and identification of HLA-A2-restricted tumor antigen peptides

(1) Establishment of Cytotoxic T Lymphocyte (CTL) Cell Line against Esophageal Cancer Cell Line

[0069] According to the disclosure of Nakao *et al.*, *Cancer Res.*, **55**:4248-4252 (1995), CTL against an esophageal cancer cell line, KE-4, belonging to squamous cell carcinomas when classified on the basis of the tissue type was established from peripheral blood monocytes of a patient, named KE-4CTL, and used in experiments. The esophageal cancer cell line KE-4 and KE-4CTL have been deposited at The National Institute of Bioscience and Human Technology (1-1-3 Higashi, Tsukuba, Ibaraki, Japan) under International Deposition Nos. FERM BP-5955 and FERM BP-5954, respectively, both on May 23, 1997. Furthermore, typing of HLA class I molecules of KE-4 was conducted according to the above-noted disclosure of Nakao *et al.*, and it was confirmed that they are HLA-A2402, -A2601, B54, -B60, -Cw1, and -Cw3.

(2) Preparation of HLA-A2601 cDNA and HLA-A2402 cDNA

[0070] Using KE-4, a recombinant plasmid was prepared by incorporating cDNA for HLA-A2601 into an expression vector pCR3 (INVITROGEN) according to the disclosure of Nakao *et al.*, *Cancer Res.*, 55:4248-4252 (1995). Another recombinant plasmid for HLA-A2402 was also prepared in the similar manner.

(3) Preparation of cDNA Library derived from KE-4

[0071] Poly $(A)^+$ mRNA was prepared from KE-4 by isolation of total RNA fraction and purification on oligo (dT) column using mRNA Purification system (manufactured by Pharmacia Biotech) according to the manufacturer's protocol. cDNAs having *Not* I adapter and *Sca* I adapter linked to each terminus were prepared from mRNAs using SuperScript™ Plasmid System (Gibco BRL) according to the manufacturer's protocol, and then ligated to an expression vector, plasmid pSV-SPORT1 (Gibco BRL), digested with restriction enzymes *Not* I and *Sal* I, to yield recombinant plasmids. The recombinant plasmids were introduced into E. coli. ElectroMAX DH10B/p3™ cells (Gibco BRL) using electric pulses in Gene Pulser (Bio-Rad) under conditions of 25 μF and 2.5 kV. Transformants into which the recombinant plasmids had been introduced were selected in LB medium (1% bacto-trypton, 0.5% yeast extract, 0.5% NaCl, pH7.3) containing ampicillin (50 μg/ml).

(4) Screening of Tumor Antigen Protein Gene

[0072] The recombinant plasmid DNAs were recovered from pools of about 100 transformants described in the above (3) as follows. A hundred transformants were introduced and cultured in each well of 96-well U-bottomed microplate containing LB medium plus ampicillin (50 μg/ml). Part of the culture was then transferred to another 96-well U-bottomed microplate containing 0.25 ml per well of TYGPN medium (F.M. Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.), and cultured for 48 hours at 37°C. The remaining cultures in LB medium on the microplate were stored in frozen. Preparation of recombinant plasmid DNAs from transformants cultured in TYGPN medium was achieved in the microplate by alkaline lysis (F.M. Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.). The recombinant plasmid DNAs recovered by isopropanol precipitation were suspended in 50 μl of 10 mM Tris, 1 mM EDTA, pH 7.4, containing 20 ng/ml RNase.

[0073] Fibroblast cell line, VA-13 cells (RIKEN CELL BANK, The Institute of Physical and Chemical Research; *Ann. Med. Exp. Biol. Fenn.*, 44:242-254, 1966) were doubly transfected with the recombinant plasmid for KE-4 cDNA and the recombinant plasmid for HLA-A2601 cDNA using Lipofectin method as follows. Seven thousands VA-13 cells were placed in each well of 96-well flat-bottomed microplate, and incubated for 2 days in 100 μl of RPMI 1640 medium containing 10% FCS. Using Lipofectin reagent (Gibco BRL), 30μl of 70 μl mixture consisting of 25 μl of the recombinant plasmid for KE-4 cDNA corresponding to about 100 transformants, 10 μl (200 ng) of the recombinant plasmid for HLA-A2601 cDNA described in (2) of Reference Example, and 35 μl of about 35-fold diluted Lipofectin reagent was added to VA-13 cells to be doubly transfected. Transfectants were prepared in duplicate. After 5 hours, 200 μl of culture medium containing 10% FCS was added to the transfectants, and further incubated for 72 hours at 37°C. After removing the culture medium, 10,000 KE-4CTL cells were added to each well, and cultured for 24 hours at 37°C in 100 μl of culture medium containing 10% FCS and 25 U/ml IL-2. The culture medium was recovered, and the amount of IFN-γ in the culture supernatant produced by KE-4CTL was measured by ELISA. Specifically, an anti-human IFN-γ mouse monoclonal antibody was adsorbed on wells of 96-well microplate as a solid-phased antibody, and after blocking non-specific bindings with bovine serum albumin, allowed to bind with IFN-γ in the above-described culture supernatant. Anti-human IFN-γ rabbit polyclonal antibody as a detection antibody was then allowed to bind, and after binding with an anti-rabbit immunoglobulin goat antibody labeled with alkaline phosphatase, reacted with para-nitrophenyl phosphate as a chromogenic substrate. After quenching the reaction by adding an equal volume of 1N NaOH, absorbance at 405 nm was measured. The absorbance was compared with that obtained with standard IFN-γ to determine the amount of IFN-γ in the supernatant.

[0074] Regarding four groups in which high production of IFN-γ was observed, corresponding frozen-stored pools of about 100 transformants containing recombinant plasmids for KE-4 cDNA were used in the following screening. The pools of the transformants were plated on LB agar medium containing ampicillin (50 μg/ml) to obtain colonies. Two hundreds colonies for each group (total 800 colonies) were cultured as described above so that a single kind of transformant is included in each well, thereby recombinant plasmid DNAs for KE-4 cDNA were prepared. Then, VA-13 cells were doubly transfected with the recombinant plasmid for KE-4 cDNA and the recombinant plasmid for HLA-A2601 cDNA followed by cocultivation with KE-4CTL, and IFN-γ produced due to KE-4CTL reaction was quantitatively determined as described above in order to select positive plasmids. In this manner, a single KE-4 cDNA recombinant plasmid clone was selected and named 6DI. Furthermore, similar procedures were repeated with 6DI to determine the amount of IFN-γ produced by KE-4CTL according to the same method as that described above. The results are shown in the following TABLE 1.

TABLE 1

Target cell	Amount of IFN-γ produced by KE-4CTL (pg/ml)
VA-13 cell	0
VA-13 cell + HLA-A2601	1.8
VA-13 cell + 6DI	4.3
VA-13 cell + HLA-A2601 + 6DI	24.0
VA-13 cell + HLA-A0201[1)]	0.9
VA-13 cell + HLA-A0201 + 6DI[1)]	3.0

[1)] For comparison, HLA of different type was transfected. (These date was obtained by transfection using the following amounts of DNA: 200 ng of HLA-A2601 or HLA-A0201, 100 ng of 6DI.)

(5) Expression Analysis for Tumor Antigen Protein Gene by Northern Hybridization

[0075] RNAs were prepared from various cell lines using RNAzol B (TEL-TEST, Inc.). Five μg of RNA was denatured in the presence of formamide and formaldehyde, electrophoresed on agarose, then transferred and fixed onto Hybond-N+ Nylon membrane (Amersham). As RNAs from normal tissues, commercially available membranes (Clontech) onto which mRNAs have been preblotted were used. The inserted sequence region of the recombinant plasmid 6DI cloned in (4) of Reference Example was labeled with ^{32}P using Multiprime DNA labeling system (Amersham) to prepare DNA probe. According to the known method (Nakayama *et al.*, *Bio-Jikken-Illustrated*, vol. 2, "Idenshi-Kaiseki-No-Kiso (A Basis for Gene Analysis)", pp. 148-151, Shujunsha, 1995), this probe was allowed to hybridize to RNAs on the membranes, and subjected to autoradiography to detect mRNA for tumor antigen protein gene of the present invention. The membranes used for the detection of mRNA for said gene were boiled in 0.5% aqueous sodium dodecyl sulfate to remove the probe, and subjected to Northern hybridization in a similar manner using β-actin as a probe which is constitutively expressed in cells, in order to detect mRNA which was used as an internal standard. The results are shown in Fig. 1. It became apparent from these results that mRNA for tumor antigen protein gene of the present invention is widely expressed in various cancer cells and normal tissues, and is about 2.5 kb in full length (Fig. 1).

(6) Cloning and Base Sequencing of Full-Length cDNA Clone Encoding Tumor Antigen Protein

[0076] KE-4-derived cDNA Library described in the above (3) of Reference Example was plated on LB agar medium containing ampicillin (50μg/ml). The colonies thus obtained were then transferred and fixed on Hybond-N+ nylon membrane (Amersham) according to the manufacturer's protocol. The same 6DI probe as that used in (5) of Reference Example was employed for hybridization and autoradiography under the same conditions as those used in (5) of Reference Example, in order to select colonies which contain recombinant plasmids having the cDNA for tumor antigen protein gene incorporated. Furthermore, recombinant plasmids were recovered from the colonies selected, treated with restriction enzymes *Not* I and *Sal* I, and then electrophoresed on agarose to determine the length of incorporated cDNAs. A recombinant plasmid incorporating cDNA of about 2.5 kb was selected, and named K3. The base sequence of the cDNA region in this plasmid K3 was determined using DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer). The base sequence thus determined is shown in SEQ ID NO: 2. The full-length of the cDNA was 2527 base pairs. The amino acid sequence (800 amino acids) encoded by the base sequence of SEQ ID NO: 2 is shown in SEQ ID NO: 1.

[0077] The analysis indicated that the base sequence shown in SEQ ID NO: 2 does not share sequence homology with known tumor antigen protein genes derived from melanomas and proved to be a different gene. The search for the base sequence of SEQ ID NO: 2 using WWW Entrez database revealed that part of the base sequence exhibits high homology of more than 90% to three gene sequences, functions thereof are unknown, decoded by WashU-Merck EST Project and registered at GENBANK under Accession Nos. R89163, R62890, and R00027. R89163, R62890 and R00027 correspond to the sequences at positions 1893-2267; 2018-2389; and 2024-2510, respectively of SEQ ID NO: 2. These three sequences, however, are those located at 3' to the initiation codon in the base sequence shown in SEQ

ID NO: 2, and therefore, the corresponding amino acid sequences cannot be determined.

[0078] After determination of the base sequence as described above, the plasmid K3 was introduced into *E. coli* JM109 to obtain *E. coli* JM109(K3) which is a transformant for storage containing the novel tumor antigen protein cDNA. *E. coli* JM109(K3) has been deposited at The National Institute of Bioscience and Human Technology (1-1-3 Higashi, Tsukuba, Ibaraki, Japan) under International Deposition No. FERM BP-5951 on May 22, 1997.

[0079] Furthermore, cDNA library (GIBCO BRL, Inc.) derived from normal human tissue (peripheral blood lymphocyte) was also screened in the manner as described above. The screening resulted in cloning of a recombinant plasmid into which cDNA of about 2.5 kb has been incorporated. When the base sequence was determined, the said cDNA thus cloned was identical with that shown in SEQ ID NO: 2 except for the base at 812 (position 812 for normal human tissue was T). This indicates that in connection with the full-length gene comprising the gene encoding the tumor antigen protein shown in SEQ ID NO: 2, almost the same genes are expressed in both cancer cells and normal human tissue.

[0080] VA-13 Cells were then doubly transfected with the recombinant plasmid K3 containing cDNA for the novel tumor antigen protein gene and another recombinant plasmid containing cDNA for HLA-A2601, and used as target cells as described in the above (4). The amount of IFN-γ produced by the reaction of KE-4CTL was determined according to the method as described in the above (4). The results are shown in the following TABLE 2.

TABLE 2

Target cell	Amount of IFN-γ produced by KE-4CTL[1)] (pg/ml)
VA-13 cell + HLA-A2601 + K3	1439
VA-13 cell + HLA-A0201[2)] + K3	10

[1)] Values obtained by subtracting the amount (background) of IFN-γ produced by KE-4CTL in response to VA-13 cells transfected with each HLA.

[2)] For comparison, HLA of different type was transfected. (These date was obtained by transfection of the following amounts of DNA: 200 ng of HLA-A2601 or HLA-A0201, 100 ng of K3.)

[0081] Based on the above results, it was confirmed that the obtained cDNA encoded a tumor antigen protein.

(7) Identification of Tumor Antigen Peptide

[0082] From the recombinant plasmid 6DI cloned in the above (4) into which partial cDNA of the novel tumor antigen protein gene have been incorporated, plasmids containing partial cDNA of various length prepared through the deletion from tumor antigen protein gene using Deletion Kit for Kilo-Sequence (Takara Shuzo Co.) according to the manufacturer's protocol were obtained. These plasmids were introduced into *E. coli* ElectroMax DH10B/p3™ cells (Gibco BRL). The cells were plated on agar medium, and 50 colonies were selected at random. From the colonies, plasmid DNAs were prepared, subjected to electrophoresis, and 5 clones which contained plasmids having appropriate length selected.

[0083] According to the method described in the above (4), VA-13 cells were doubly transfected with HLA-A2601 cDNA and the above plasmid DNA, cocultured with KE-4CTL, and IFN-γ in the culture medium was quantitatively determined according to the method described in (4). As a result, it was found that the transfectant with a plasmid lacking the base sequence after position 2253 in SEQ ID No:2 had no IFN-γ-inducing activity on KE-4CTL. It was therefore suggested that peptides having the sequence after about position 739 in the amino acid sequence of SEQ ID NO: 1 may have IFN-γ-inducing activity on KE-4CTL.

[0084] Thus, a series of 21 different peptides each consisting of successive 10 amino acid residues in the amino acid sequence after position 730 in SEQ ID NO: 1 were synthesized so that they each have the amino acid sequence shifted serially by three amino acid residues. Using these peptides, IFN-γ in culture medium was determined as described above except that the antigen presentation was achieved by pulsing HLA-A2601 cDNA-transfected VA-13 cells with the peptides. As the result, IFN-γ-inducing activity was observed in the peptides having the amino acid sequences of positions 736 to 745 (736-745), positions 748 to 757 (748-757), and positions 784 to 793 (784-793) in SEQ ID NO: 1.

[0085] For each of these three peptides, additional peptides consisting of 9 amino acid residues were synthesized by truncating the N- or C-terminal residue, and used for measurement of IFN-γ-inducing activity in a similar manner. Stronger IFN-γ-inducing activity was observed for the peptides having the amino acid sequences of positions 736 to 744 (736-744), positions 749 to 757 (749-757), and positions 785 to 793 (785-793) in SEQ ID NO: 1. The results are shown in TABLE 3.

TABLE 3

Pulsed cell	Peptide	Amount of INF-γ produced by KE4-CTL cells (pg/ml)
VA-13/A2601[1)]	"736-744"	203
VA-13/A0201[2)]	"736-744"	44
VA-13/A2601	"749-757"	183
VA-13/A0201	"749-757"	89
VA-13/A2601	"785-793"	394
VA-13/A0201	"785-793"	102

1) VA-13 cells transfected with HLA-A2601 cDNA
2) VA-13 cells transfected with different HLA-A0201 cDNA as a control

[0086] The results in TABLE 3 indicate that these peptides function as HLA-A24-restricted tumor antigen peptides.

[0087] HLA-A24-restricted tumor antigen peptides were then identified as follows.

[0088] There are certain rules (motifs) in the sequences of antigen peptides bound and presented by HLA molecules. Concerning the motif for HLA-A24, it is known that in the sequence of peptides consisting of 8 to 11 amino acids, the amino acid at the second position from the N-terminus is phenylalanine, tyrosine, methionine, or tryptophan, and the amino acid at the C-terminus is phenylalanine, leucine, isoleucine, tryptophan, or methionine (*Immunogenetics*, **41**:178-228, 1995; *J. Immunol.*, **152**: 3913, 1994; *J. Immunol.*, **155**:4307, 1994).

[0089] Thus, another peptide consisting of the segment from position 690 to position 698 (690-698; SEQ ID NO: 3) in SEQ ID NO: 1 which corresponds to the above motif was further synthesized. VA-13 cells transfected with HLA-A2402 cDNA was then pulsed with said peptide, and IFN-γ-inducing activity on KE-4CTL was measured as described above. The results are shown in TABLE 4.

TABLE 4

Pulsed cell	Peptide	Amount of INF-γ produced by KE4-CTL cells (pg/ml)
VA-13	"690-698"	157
VA-13/A2402[1)]	"690-698"	269
VA-13/A0201[2)]	"690-698"	166

1) VA-13 cells transfected with HLA-A2402 cDNA
2) VA-13 cells transfected with different HLA-A0201 cDNA as a control

[0090] The results in TABLE 4 suggest that the peptide "690-698 (SEQ ID NO: 3)" functions as a tumor antigen peptide.

(8) Induction of CTL from peripheral blood lymphocytes by tumor antigen peptides

[0091] The inventors have investigated whether antigen-specific CTL can be induced from peripheral blood lym-

phocytes of the cancer patient from whom KE-4 was derived, by *in vitro* stimulation with the tumor antigen peptides described in the above (7). Tumor antigen peptides used were those having the sequences of "736-744", "749-757", and "690-698", obtained above in (7) of Reference Example. Frozen peripheral blood lymphocytes, which had been separated from the above cancer patient using Ficoll method, were awoke, transferred to 24-well plate at about $2x10^6$ cells/well, and cultured in RPMI 1640 medium containing 10% FCS and IL-2 (100 U/ml). To stimulate the peripheral blood lymphocytes, the above tumor antigen peptide was added to the culture medium at 10 μg/ml. One week later, 10 μg/ml of the above tumor antigen peptide was added together with about $1x10^5$ cells of X ray-radiated (50 Gy) peripheral blood lymphocytes for the second stimulation. After additional one week, the third stimulation was conducted in a similar manner.

[0092] As for peptides having the sequences of "736-744" and "749-757", peripheral blood lymphocytes were recovered one week after the third stimulation, and measured for their cytotoxic activity (specific lysis) using, as target cells, ^{51}Cr-labeled KE-4 and another esophageal cancer cell line KE-3 of which HLA-A loci are A2402 and A2, according to the method described in D.D. Kharkevitch *et al.*, *Int. J. Cancer*, **58**:317 (1994). The results are shown in TABLE 5.

TABLE 5

Effector cell	Target cell	Specific lysis (%)
Peripheral blood lymphocytes stimulated with "736-744"	KE-4	22.1
	KE-3	3.7
Peripheral blood lymphocytes stimulated with "749-757"	KE-4	35.9
	KE-3	24.2

[0093] When stimulated with the peptide having the sequence of "736-744", KE-4 was severely injured, whereas the negative control KE-3 was not injured. It was therefore demonstrated that CTL specific for KE-4 was induced. Similarly, when stimulated with the peptide having the sequence of "749-757", stronger cytotoxic activity was observed on KE-4, although nonspecific cytotoxic activity was also observed on KE-3, indicating that CTL specific for KE-4 was induced.

[0094] For peptide having the sequence of "690-698 (SEQ ID NO: 3)", peripheral blood lymphocytes were recovered after the third stimulation, and further cultured in RPMI-1640 medium containing 10% FCS, 50% AIM-V (Gibco BRL), and IL-2 (100 U/ml). Then, the cytotoxic activity was measured as above using ^{51}Cr-labeled KE-4 and VA-13 cells as target cells. In addition, lymphocytes were isolated from peripheral blood of a normal individual of which HLA-A loci were homozygous A24, and measured for their cytotoxic activity (specific lysis) in the same manner as above using, as target cells, ^{51}Cr-labeled KE-4 and lung cancer cell line QG-56 of which HLA-A loci are homozygous A2601. The results are shown in TABLE 6.

TABLE 6

Effector cell	Target cell	Specific lysis (%)
"690-698"-Stimulated peripheral blood lymphocytes from a cancer patient	KE-4	24.7
	VA-13	13.8
"690-698"-Stimulated peripheral blood lymphocytes from a normal individual	KE-4	17.7
	QG-56	11.5

[0095] When peripheral blood lymphocytes from a cancer patient and from a normal individual were stimulated with the peptide having the sequence of "690-698 (SEQ ID NO: 3)", stronger cytotoxic activity was observed on KE-4, although nonspecific cytotoxic activity was also observed on the negative controls VA-13 and QG-56 cells. The above results indicate that CTLs specific for KE-4 were induced.

Example 1 Synthesis of Tumor Antigen Peptide Derivatives

[0096] As described above, there are certain rules (motifs) in the sequences of antigen peptides bound and presented by HLA molecules. Concerning the motif for HLA-A24, it is known that in the sequence of peptides consisting of

8 to 11 amino acids, the amino acid at the second position from the N-terminus is phenylalanine, tyrosine, methionine, or tryptophan, and the amino acid at the C-terminus is phenylalanine, leucine, isoleucine, tryptophan, or methionine (*Immunogenetics*, **41**:178-228, 1995; *J. Immunol.*, **152**: 3913, 1994; *J. Immunol.*, **155**:4307, 1994). With regard to the peptide "690-698 (SEQ ID NO: 3)" which was identified as an HLA-A24-restricted tumor antigen peptide in the foregoing (7) and (8) of Reference Example, amino acid sequences of peptide derivatives containing amino acid substitution(s) according to the above motif are shown in SEQ ID NO: 4.

[0097] A variety of such tumor antigen peptide derivatives were prepared in which the second and/or ninth amino acid residue(s) of the HLA-A24-restricted tumor antigen peptide consisting of the amino acid sequence shown in SEQ ID NO: 3 were altered on the basis of the above-mentioned rules.

[0098] The following are several specific examples:

a) Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp-Trp,
b) Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp-Leu,
c) Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp-Ile,
d) Glu-Phe-Arg-Gly-Phe-Thr-Gln-Asp-Phe,
e) Glu-Phe-Arg-Gly-Phe-Thr-Gln-Asp-Trp

[0099] These peptides were synthesized by the Fmoc method using Advanced Chemtech MPS 350, and then purified by HPLC using YMC-Pack ODS-A SH-363-5 column. The purified products were all at or greater than 95% purity.

[0100] The method of synthesizing peptide derivatives are described below in more detail in reference to the exemplary peptides:

(1) Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp-Ile (SEQ ID NO: 5);
(2) Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp-Leu (SEQ ID NO: 6); and
(3) Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp-Trp (SEQ ID NO: 7).

(1) Synthesis of Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp-Ile (SEQ ID NO: 5)

[0101] Fmoc-Ile-Alko Resin (0.62 mmol/g, 100-200 mesh) was used as the resin. Using 100 mg of this resin, the synthesis was started according to Schedule 1 described below in TABLE 7 to couple the following residues in order: Fmoc-Asp(OtBu), Fmoc-Gln-OH, Fmoc-Thr(tBu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Glu(OtBu)-OH. After the completion of coupling, the procedures were carried out up to Step 3 of Schedule 1 to obtain the peptide resin.

[0102] To this peptide resin, 2 ml of Reagent K (5% phenol, 5% thioanisole, 5% H_2O, and 2.5% ethanedithiol in TFA) was added and allowed to react for 2.5 hours at room temperature. To the resin was added 10 ml of diethyl ether under cooling with ice, and the mixture was stirred for 10 minutes, filtered, and then washed with 10 ml of diethyl ether. To the filter cake, 10 ml of aqueous acetic acid was added and the mixture was stirred for 30 minutes. The resin was then filtered off, and washed with 4 ml of aqueous acetic acid. After lyophilizing the filtrate and washing, the resultant crude peptide was dissolved in aqueous acetic acid, and loaded onto a reverse phase packing material YMC-Pack ODS-A SH-363-5 column (30 ϕ x 250 mm) pre-equilibrated with 0.1% aqueous TFA. The column was washed with 0.1% aqueous TFA, and eluted while increasing the concentration of acetonitrile up to 24% over 200 minutes at a flow rate of 7 ml/min. The eluate was monitored at A 220 nm. The fractions containing the desired product were combined together and lyophilized to obtain 47.8 mg of Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp-Ile.

[0103] The resultant peptide Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp-Ile had a retention time of 19.3 minutes when analyzed by a reverse phase packing material YMC-PACK ODS-AM AM-303 column (4.6 ϕ x 250 mm) eluting with 0 to 60 % linear gradient of acetonitrile containing 0.1% TFA. The amino acid analysis and mass spectrometry of the said peptide were consistent with the theoretical values.

Amino Acid Analysis

[0104]

Hydrolysis: 1% phenol/6N aqueous hydrochloric acid, 110°C, 24 hours;
Analytical method: the ninhydrin method; *: reference amino acid; theoretical values are indicated in parentheses.

Asx: 0.94 (1)
Thr: 0.91 (1)
Glx: 1.94 (2)

Gly: 0.99 (1)
*Ile: 1.00 (1)
Tyr: 0.93 (1)
Phe: 0.98 (1)
Arg: 0.95 (1)

Mass spectrum (FAB): [M+H]+: 1128

TABLE 7

Schedule 1		
Step		(min) x times*
1. washing	DMF 1.2 ml	1 x 2
2.deprotection	50% piperidine/DMF	12 x 1
3.washing	DMF 1.2 ml	1 x 7
4.coupling	an amino-protected amino acid (5 eq.) /NMP solution 0.9 ml, DIC (5 eq.)/NMP solution 0.3 ml	30 x 1
5.washing	DMF 1.2 ml	1 x 2
6.coupling	an amino-protected amino acid (5 eq.) /NMP solution 0.9 ml, DIC (5 eq.)/NMP solution 0.3 ml	30 x 1
7.washing	DMF 1.2 ml	1 x 4

*: Duration (min) x the number of times of treatment

(2) Synthesis of Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp-Leu (SEQ ID NO: 6)

[0105] In the same manner as that of the foregoing section (1), using 100 mg of Fmoc-Leu-Alko Resin (0.54 mmol/g, 100-200 mesh), Fmoc-Asp (OtBu)-OH, Fmoc-Gln-OH, Fmoc-Thr(tBu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Glu(OtBu)-OH were coupled in order, and the product was then deprotected. The resultant crude peptide was dissolved in aqueous acetic acid and loaded onto a reverse phase packing material YMC-Pack ODS-A SH-363-5 column (30 ϕ x 250 mm) pre-equilibrated with 0.1% aqueous TFA. The column was washed with 0.1% aqueous TFA, and eluted while increasing the concentration of acetonitrile up to 25% over 200 minutes at a flow rate of 7 ml/min. The eluate was monitored at A 220 nm. The fractions containing the desired product were combined and lyophilized to obtain 52.5 mg of Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp- Leu.

[0106] The resultant peptide Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp-Leu had a retention time of 19.6 minutes when analyzed by a reverse phase packing material YMC-PACK ODS-AM AM-303 column (4.6 ϕ x 250 mm) eluting with 0 to 60% linear gradient of acetonitrile containing 0.1% TFA. The amino acid analysis and mass spectrometry of the said peptide were consistent with the theoretical values.

Amino Acid Analysis

[0107]

Hydrolysis: 1% phenol/6N aqueous hydrochloric acid, 110°C, 24 hours; analytical method: the ninhydrin method; *: reference amino acid; theoretical values are indicated in parentheses.

Asx: 0.97 (1)
Thr: 0.94 (1)
Glx: 1.98 (2)
Gly: 1.02 (1)
*Leu: 1.00 (1)
Tyr: 0.94 (1)
Phe: 1.00 (1)
Arg: 0.97 (1)

Mass spectrum (FAB): [M+H]+: 1128

(3) Synthesis of Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp-Trp (SEQ ID NO: 7)

[0108] In the same manner as that of the foregoing section (1), using 100 mg of Fmoc-Trp(Boc)-Alko Resin (0.65 mmol/g, 100-200 mesh), Fmoc-Asp (OtBu)-OH, Fmoc-Gln-OH, Fmoc-Thr(tBu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Glu(OtBu)-OH were coupled in order, and the product was then deprotected. The resultant crude peptide was dissolved in aqueous acetic acid and loaded onto a reverse phase packing material YMC-Pack ODS-A SH-363-5 column (30 ϕ x 250 mm) pre-equilibrated with 0.1% aqueous TFA. The column was washed with 0.1% aqueous TFA, and eluted while increasing the concentration of acetonitrile up to 26% over 200 minutes at a flow rate of 7 ml/min. The eluate was monitored at A 220 nm. The fractions containing the desired product were combined and lyophilized to obtain 14.0 mg of Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp- Trp.

[0109] The resultant peptide Glu-Tyr-Arg-Gly-Phe-Thr-Gln-Asp- Trp had a retention time of 20.7 minutes when analyzed using a reverse phase packing material YMC-PACK ODS-AM AM-303 column (4.6 ϕ x 250 mm) eluting with 0 to 60% linear gradient of acetonitrile containing 0.1% TFA. The amino acid analysis (Trp could not be detected) and mass spectrometry of the said peptide were consistent with the theoretical values.

Amino Acid Analysis

[0110]

Hydrolysis: 1% phenol/6N aqueous hydrochloric acid, 110°C, 24 hours; analytical method: the ninhydrin method; *: reference amino acid; theoretical values are indicated in parentheses.

Asx: 0.67 (1)
Thr: 0.96 (1)
Glx: 2.00 (2)
Gly: 1.02 (1)
Tyr: 0.96 (1)
*Phe: 1.00 (1)
Arg: 1.01 (1)

Mass spectrum (FAB): [M+H]+: 1202

Example 2 Activity Measurement of Tumor Antigen Peptide Derivatives

[0111] The peptides prepared in Example 1 can be examined for the IFN-γ-inducing activity as described in (7) of Reference Example or the CTL-inducing ability as described in (8) of Reference Example so as to demonstrate that these peptides have functions as a tumor antigen peptide. An example is shown below.

[0112] Peripheral blood lymphocytes obtained from an HLA-A24-positive healthy human were subjected to *in vitro* peptide stimulation in the same manner as Reference Example (8) using the three tumor antigen peptide derivatives (SEQ ID NOs: 5-7) synthesized in the above Example 1 to examine whether or not the peptides can induce CTLs. The peripheral blood lymphocytes were recovered one week after the third stimulation with the peptides, and the amount of IFN-γ produced by the peripheral blood lymphocytes in response to the stimulation was measured using, as target cells, HLA-A24-positive KE-4 cells expressing the tumor antigen. Separately, the amount of IFN-γ produced by the peripheral blood lymphocytes in response to the stimulation was measured using, as target cells , HLA-A24-negative VA-13 cells in a similar and used as the background value. The antigen-specific CTL activity was calculated by subtracting the background amount of IFN-γ produced against VA-13 cells from the amount of IFN-γ produced against KE-4 cells. The results are shown in Fig. 2. It was demonstrated that CTLs were induced by each of the peptide derivatives shown in SEQ ID NOs: 5, 6, and 7. In particular, the tumor antigen peptide derivative shown in SEQ ID NO: 5 exhibited a strong activity of inducing IFN-γ production.

[0113] Alternatively, the same activity measurement as that described above may also be carried out using commercially available SKG-IIIa cells (JCR B0232) instead of KE-4 cells as tumor antigen- and HLA-A24-positive target cells.

INDUSTRIAL APPLICABILITY

[0114] The novel tumor antigen peptide derivatives provided by the present invention are useful in prophylaxis,

treatment, or diagnosis of a wide range of tumors.

SEQUENCE LISTING FREE TEXT

[0115] In the amino acid sequence shown in SEQ ID NO: 4, the second amino acid is phenylalanine, tyrosine, methionine, or tryptophan, and the ninth amino acid is phenylalanine, leucine, isoleucine, tryptophan, or methionine.

SEQUENCE LISTING

<110> ITOH, Kyogo

<120> Tumor Antigen Peptide Derivatives

70

<130> 661092

<160> 7

<210> 1

<211> 800

<212> PRT

<213> Homo sapiens

<400> 1

Met Gly Ser Ser Lys Lys His Arg Gly Glu Lys Glu Ala Ala Gly Thr
5 10 15

Thr Ala Ala Ala Gly Thr Gly Gly Ala Thr Glu Gln Pro Pro Arg His
20 25 30

Arg Glu His Lys Lys His Lys His Arg Ser Gly Gly Ser Gly Gly Ser
35 40 45

Gly Gly Glu Arg Arg Lys Arg Ser Arg Glu Arg Gly Gly Glu Arg Gly
50 55 60

Ser Gly Arg Arg Gly Ala Glu Ala Glu Ala Arg Ser Ser Thr His Gly
65 70 75 80

Arg Glu Arg Ser Gln Ala Glu Pro Ser Glu Arg Arg Val Lys Arg Glu
85 90 95

100 105 110

Gly Asp Ala Ser Ser Leu Ser Ile Glu Glu Thr Asn Lys Leu Arg Ala

115 120 125

Lys Leu Gly Leu Lys Pro Leu Glu Val Asn Ala Ile Lys Lys Glu Ala

130 135 140

Gly Thr Lys Glu Glu Pro Val Thr Ala Asp Val Ile Asn Pro Met Ala

145 150 155 160

Leu Arg Gln Arg Glu Glu Leu Arg Glu Lys Leu Ala Ala Ala Lys Glu

165 170 175

Lys Arg Leu Leu Asn Gln Lys Leu Gly Lys Ile Lys Thr Leu Gly Glu

180 185 190

Asp Asp Pro Trp Leu Asp Asp Thr Ala Ala Trp Ile Glu Arg Ser Arg

195 200 205

Gln Leu Gln Lys Glu Lys Asp Leu Ala Glu Lys Arg Ala Lys Leu Leu

210 215 220

Glu Glu Met Asp Gln Glu Phe Gly Val Ser Thr Leu Val Glu Glu Glu

225 230 235 240

Phe Gly Gln Arg Arg Gln Asp Leu Tyr Ser Ala Arg Asp Leu Gln Gly

245 250 255

Leu Thr Val Glu His Ala Ile Asp Ser Phe Arg Glu Gly Glu Thr Met

260 265 270

Ile Leu Thr Leu Lys Asp Lys Gly Val Leu Gln Glu Glu Glu Asp Val

275 280 285

Leu Val Asn Val Asn Leu Val Asp Lys Glu Arg Ala Glu Lys Asn Val

290 295 300

Glu Leu Arg Lys Lys Lys Pro Asp Tyr Leu Pro Tyr Ala Glu Asp Glu

305 310 315 320

S r Val Asp Asp Leu Ala Gln Gln Lys Pro Arg Ser Ile Leu Ser Lys
325 330 335
Tyr Asp Glu Glu Leu Glu Gly Glu Arg Pro His Ser Phe Arg Leu Glu
340 345 350
Gln Gly Gly Thr Ala Asp Gly Leu Arg Glu Arg Glu Leu Glu Glu Ile
355 360 365
Arg Ala Lys Leu Arg Leu Gln Ala Gln Ser Leu Ser Thr Val Gly Pro
370 375 380
Arg Leu Ala Ser Glu Tyr Leu Thr Pro Glu Glu Met Val Thr Phe Lys
385 390 395 400
Lys Thr Lys Arg Arg Val Lys Lys Ile Arg Lys Lys Glu Lys Glu Val
405 410 415
Val Val Arg Ala Asp Asp Leu Leu Pro Leu Gly Asp Gln Thr Gln Asp
420 425 430
Gly Asp Phe Gly Ser Arg Leu Arg Gly Arg Gly Arg Arg Arg Val Ser
435 440 445
Glu Val Glu Glu Glu Lys Glu Pro Val Pro Gln Pro Leu Pro Ser Asp
450 455 460
Asp Thr Arg Val Glu Asn Met Asp Ile Ser Asp Glu Glu Glu Gly Gly
465 470 475 480
Ala Pro Pro Pro Gly Ser Pro Gln Val Leu Glu Glu Asp Glu Ala Glu
485 490 495
Leu Glu Leu Gln Lys Gln Leu Glu Lys Gly Arg Arg Leu Arg Gln Leu
500 505 510
Gln Gln Leu Gln Gln Leu Arg Asp Ser Gly Glu Lys Val Val Glu Ile
515 520 525
Val Lys Lys Leu Glu Ser Arg Gln Arg Gly Trp Glu Glu Asp Glu Asp

530 535 540

Pro Glu Arg Lys Gly Ala Ile Val Phe Asn Ala Thr Ser Glu Phe Cys
545 550 555 560
Arg Thr Leu Gly Glu Ile Pro Thr Tyr Gly Leu Ala Gly Asn Arg Glu
565 570 575
Glu Gln Glu Glu Leu Met Asp Phe Glu Arg Asp Glu Glu Arg Ser Ala
580 585 590
Asn Gly Gly Ser Glu Ser Asp Gly Glu Glu Asn Ile Gly Trp Ser Thr
595 600 605
Val Asn Leu Asp Glu Glu Lys Gln Gln Gln Asp Phe Ser Ala Ser Ser
610 615 620
Thr Thr Ile Leu Asp Glu Glu Pro Ile Val Asn Arg Gly Leu Ala Ala
625 630 635 640
Ala Leu Leu Leu Cys Gln Asn Lys Gly Leu Leu Glu Thr Thr Val Gln
645 650 655
Lys Val Ala Arg Val Lys Ala Pro Asn Lys Ser Leu Pro Ser Ala Val
660 665 670
Tyr Cys Ile Glu Asp Lys Met Ala Ile Asp Asp Lys Tyr Ser Arg Arg
675 680 685
Glu Glu Tyr Arg Gly Phe Thr Gln Asp Phe Lys Glu Lys Asp Gly Tyr
690 695 700
Lys Pro Asp Val Lys Ile Glu Tyr Val Asp Glu Thr Gly Arg Lys Leu
705 710 715 720
Thr Pro Lys Glu Ala Phe Arg Gln Leu Ser His Arg Phe His Gly Lys
725 730 735
Gly Ser Gly Lys Met Lys Thr Glu Arg Arg Met Lys Lys Leu Asp Glu
740 745 750

Glu Ala Leu Leu Lys Lys Met Ser Ser Ser Asp Thr Pro Leu Gly Thr
755 760 765

Val Ala Leu Leu Gln Glu Lys Gln Lys Ala Gln Lys Thr Pro Tyr Ile
770 775 780

Val Leu Ser Gly Ser Gly Lys Ser Met Asn Ala Asn Thr Ile Thr Lys
785 790 795 800

<210> 2

<211> 2527

<212> DNA

<213> Homo sapiens

<220>

<221> 5'UTR

<222> (1)...(38)

<220>

<221> CDS

<222> (39)...(2438)

<220>

<221> 3'UTR

<222> (2439)...(2506)

<400> 2

ggttcggcgg cagccgggct cggagtggac gtgccactat ggggtcgtcc aagaagcatc 60

gcggagagaa ggaggcggcc gggacgacgg cggcggccgg caccgggggt gccaccgagc 120

agccgccgcg gcaccgggaa cacaaaaaac acaagcaccg gagtggcggc agtggcggta 180

gcggtggcga acgacggaag cggagccggg aacgtggggg cgagcgcggg agcgggcggc 240

gcggggccga agctgaggcc cggagcagca cgcacgggcg ggagcgcagc caggcagagc 300

cctccgagcg gcgcgtgaag cgggagaagc gcgatgacgg ctacgaggcc gctgccagct 360

ccaaaactag ctcaggcgat gcctcctcac tcagcatcga ggagactaac aaactccggg 420

caaagttggg gctgaaaccc ttggaggtta atgccatcaa gaaggaggcg ggcaccaagg 480

aggagcccgt gacagctgat gtcatcaacc ctatggcctt gcgacagcga gaggagctgc 540

gggagaagct ggcggctgcc aaggagaagc gcctgctgaa ccaaaagctg gggaagataa 600

agaccctagg agaggatgac ccctggctgg acgacactgc agcctggatc gagaggagcc 660

ggcagctgca gaaggagaag gacctggcag agaagagggc caagttactg gaggagatgg 720

accaagagtt tggtgtcagc actctggtgg aggaggagtt cgggcagagg cggcaggacc 780

tgtacagtgc ccgggacctg cagggcctca ccgtggagca tgccattgat tccttccgag 840

aaggggagac aatgattctt accctcaagg acaaaggcgt gctgcaggag gaggaggacg 900

tgctggtgaa cgtgaacctg gtggataagg agcgggcaga gaaaaatgtg gagctgcgga 960

agaagaagcc tgactacctg ccctatgccg aggacgagag cgtggacgac ctggcgcagc 1020

aaaaacctcg ctctatcctg tccaagtatg acgaagagct tgaaggggag cggccacatt 1080

ccttccgctt ggagcagggc ggcacggctg atggcctgcg ggagcgggag ctggaggaga 1140

tccgggccaa gctgcggctg caggctcagt ccctgagcac agtggggccc cggctggcct 1200

ccgaatacct cacgcctgag gagatggtga cctttaaaaa gaccaagcgg agggtgaaga 1260

aaatccgcaa gaaggagaag gaggtagtag tgcgggcaga tgacttgctg cctctcgggg 1320

accagactca ggatggggac tttggttcca gactgcgggg acggggtcgc cgccgagtgt 1380

ccgaagtgga ggaggagaag gagcctgtgc ctcagcccct gccgtcggac gacacccgag 1440

tggagaacat ggacatcagt gatgaggagg aaggtggagc tccaccgccg gggtccccgc 1500

aggtgctgga ggaggacgag gcggagctgg agctgcagaa gcagctggag aagggacgcc 1560

ggctgcgaca gttacagcag ctacagcagc tgcgagacag tggcgagaag gtggtggaga 1620

ttgtgaagaa gctggagtct cgccagcggg gctgggagga ggatgaggat cccgagcgga 1680

aggggccat cgtgttcaac gccacgtccg agttctgccg caccttgggg gagatcccca 1740

cctacgggct ggctggcaat cgcgaggagc aggaggagct catggacttt gaacgggatg 1800

aggagcgctc agccaacggt ggctccgaat ctgacgggga ggagaacatc ggctggagca 1860

cggtgaacct ggacgaggag aagcagcagc aggatttctc tgcttcctcc accaccatcc 1920

tggacgagga accgatcgtg aataggggge tggcagctgc cctgctcctg tgtcagaaca 1980

aagggctgct ggagaccaca gtgcagaagg tggcccgggt gaaggccccc aacaagtcgc 2040

tgccctcagc cgtgtactgc atcgaggata agatggccat cgatgacaag tacagccgga 2100

gggaggaata ccgaggcttc acacaggact tcaaggagaa ggacggctac aaacccgacg 2160

ttaagatcga atacgtggat gagacgggcc ggaaactcac acccaaggag gctttccggc 2220

agctgtcgca ccgcttccat ggcaagggct caggcaagat gaagacagag cggcggatga 2280

agaagctgga cgaggaggcg ctcctgaaga agatgagctc cagcgacacg cccctgggca 2340

ccgtggccct gctccaggag aagcagaagg ctcagaagac cccctacatc gtgctcagcg 2400

gcagcggcaa gagcatgaac gcgaacacca tcaccaagtg acagcgccct cccgtagtcg 2460

gccctgcctc aaccttcata ttaaataaag ctccctcctt atttttaaaa aaaaaaaaaa 2520

aaaaaaa 2527

<210> 3

<211> 9

<212> PRT

<213> Homo sapiens

<400> 3

Glu Tyr Arg Gly Phe Thr Gln Asp Phe

5

<210> 4

<211> 9

<212> PRT

<213> Artificial Sequence

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<221> VARIANT

<222> 2

<223> Xaa is Phe, Tyr, Met or Trp.

<220>

<221> VARIANT

<222> 9

<223> Xaa is Phe, Leu, Ile, Trp or Met.

<400> 4

Glu Xaa Arg Gly Phe Thr Gln Asp Xaa
5

<210> 5

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<221> VARIANT

<400> 5

Glu Tyr Arg Gly Phe Thr Gln Asp Ile
5

<210> 6

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<221> VARIANT

<400> 6

Glu Tyr Arg Gly Phe Thr Gln Asp Leu

5

<210> 7

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<221> VARIANT

<400> 7

Glu Tyr Arg Gly Phe Thr Gln Asp Trp

5

Claims

1. A tumor antigen peptide derivative which comprises all or part of an amino acid sequence wherein one to several amino acid residues in the amino acid sequence shown in SEQ ID NO: 3 are altered, and which derivative is capable of binding to HLA-A24 antigen and thus being recognized by cytotoxic T cells.

2. A tumor antigen peptide derivative of claim 1 which comprises all or part of an amino acid sequence wherein the amino acid residue or residues at the second position and/or the ninth position of the amino acid sequence shown in SEQ ID NO: 3 are substituted with another amino acid residue.

3. A tumor antigen peptide derivative of claim 2 which comprises all or part of the amino acid sequence shown in SEQ ID NO: 4.

4. A tumor antigen peptide derivative of claim 3 which comprises all or part of an amino acid sequence wherein phenylalanine at the ninth position of the amino acid sequence shown in SEQ ID NO: 3 is substituted with tryptophan, leucine, or isoleucine.

5. A tumor antigen peptide derivative of claim 3 which comprises all or part of an amino acid sequence wherein tyro-

sine at the second position of the amino acid sequence shown in SEQ ID NO: 3 is substituted with phenylalanine.

6. A tumor antigen peptide derivative of claim 3 which comprises all or part of an amino acid sequence wherein, in the amino acid sequence shown in SEQ ID NO: 3, phenylalanine at the ninth position is substituted with tryptophan, leucine, or isoleucine, and tyrosine at the second position is also substituted with phenylalanine.

7. A tumor antigen peptide derivative of claim 4 which comprises all or part of the amino acid sequence shown in SEQ ID NO: 5.

8. A therapeutic or prophylactic agent for tumors which comprises at least one tumor antigen peptide derivative selected from those derivatives set forth in claims 1-7.

9. An antigen-presenting cell which comprises a complex between HLA-A24 antigen and a tumor antigen peptide derivative according to any one of claims 1-7, the complex being presented on the surface of isolated cell having an antigen-presenting ability derived from a tumor patient.

10. A therapeutic agent for tumors which comprises the antigen-presenting cell of claim 9 as an active ingredient.

11. A cytotoxic T cell which specifically recognizes a complex between HLA-A24 antigen and a tumor antigen peptide derivative according to any one of claims 1-7.

12. A therapeutic agent for tumors which comprises the cytotoxic T cell of claim 11 as an active ingredient.

Fig. 1

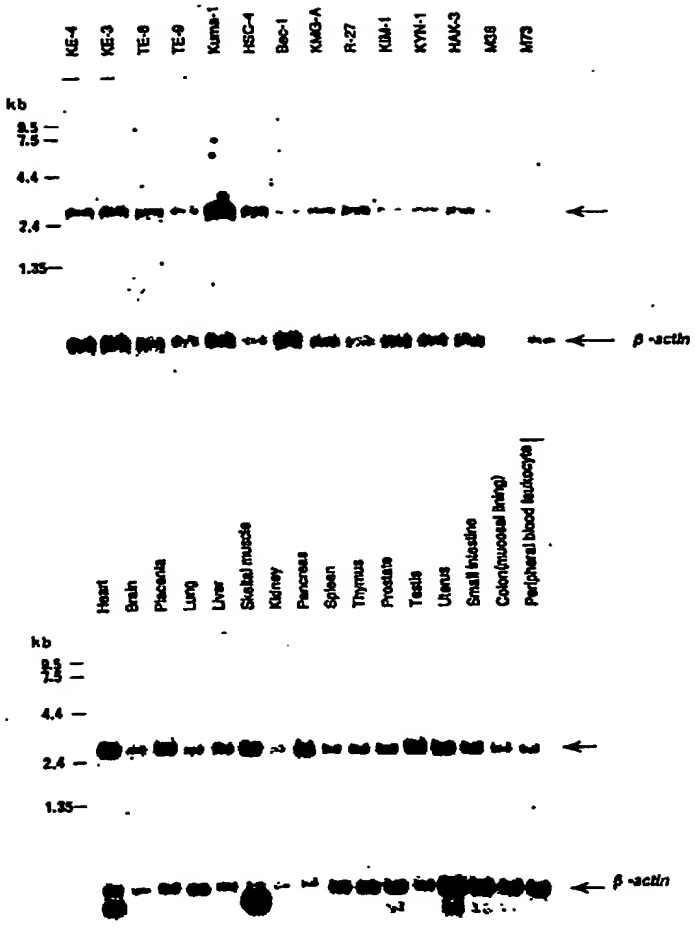
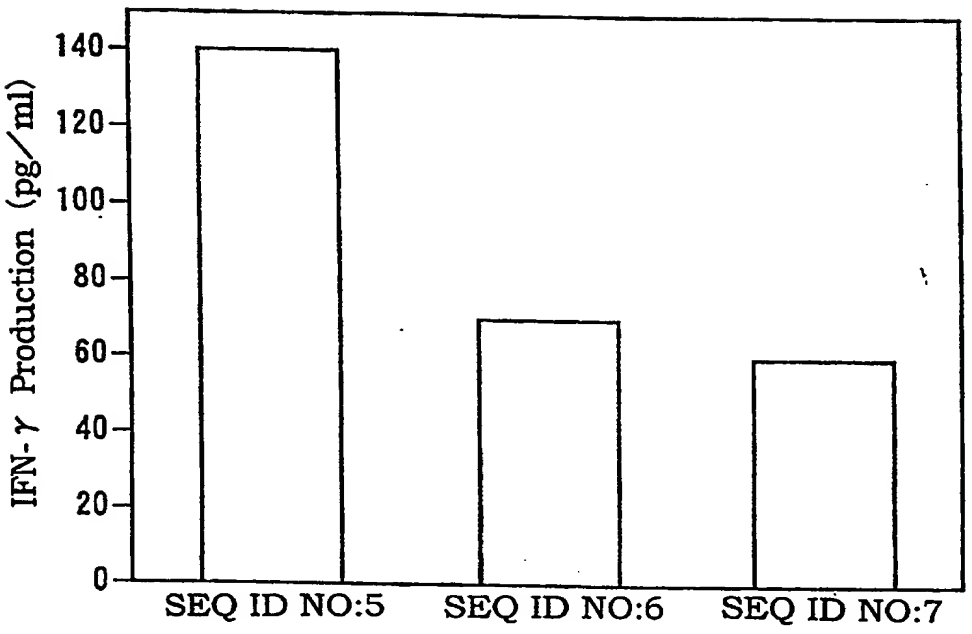

KE-4
KE-3
TE-8
TE-9
Kuma-1
HSC-4
Bec-1
KMG-A
R-27
KM-1
KYN-1
HAK-3
M38
M79
kb
7.5
4.4
2.4
1.35
β-actin
Heart
Brain
Placenta
Lung
Liver
Skeletal muscle
Kidney
Pancreas
Spleen
Thymus
Prostate
Testis
Uterus
Small intestine
Colon(mucosal lining)
Peripheral blood leukocyte
kb
4.4
2.4
1.35
β-actin

Fig. 2


IFN-γ Production (pg/ml)
140
120
100
80
60
40
20
0
SEQ ID NO:5
SEQ ID NO:6
SEQ ID NO:7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/05430

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[6] C07K7/06, C12N5/08, 15/12, A61K38/08, 45/05

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[6] C07K7/06, 14/82, C12N5/06, 5/08, 15/12-15/28, A61K38/08, 38/17

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPI (DIALOG), BIOSIS (DIALOG), CA (STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y A	Cancer Research, Volume 55, issued 1 October 1995, Masanobu Nakao et al., "HLA A2601-restricted CTLs Recognize a Peptide Antigen Expressed on Squamous Cell Carcinoma", pages 4248-4252	9, 11 10, 12 1-8
Y A	Clinical Immunology, Vol. 27, No. 9, September, 1995, Yuuko Yamazaki, "Gairai Kougen no MHC class I ni yoru teiji no kijo", p.1034-1042	10, 12 1-9, 11
PX	WO, 97/46676, A1 (Kyogo Itoh), 11 December, 1997 (11. 12. 97) & AU, 9730479, A	1-8
PX	Journal of Experimental Medicine, Volume 187, Number 3, 2 February 1998, Shigeki Shichijo et al., "A Gene Encoding Antigenic Peptides of Human Squamous Cell Carcinoma Recognized by Cytotoxic T Lymphocytes", pages 277-288	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 March, 1999 (02. 03. 99)	16 March, 1999 (16. 03. 99)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)